cAMP Assay

This invention relates to a method for assaying for
cyclic adenosine monophosphate (cAMP) and other cyclic
nucleotides and cyclic nucleotide analogs and to kits
for use in such methods.

cAMP or adenosine 3',5'-cyclic monophosphate
comprises a ribofuranose monosaccharide unit with a
purine group attached at the 1' position and a phosphate
group attached at the 3' and 5' positions. cAMP is
formed by cyclization of ATP and is a "second messenger"
which functions as a mediator of hormone action for a
range of hormones. In particular it serves to activate
key enzymes known as protein kinases.

cAMP is widely distributed in the body and has been
assayed for in connection with a broad range of
conditions, eg thyroid malfunction, calcium metabolism
disorders, etc.

Several assays for cAMP are available commercially,
especially from Amersham plc, UK. The most widely used
assays are based on competition between cAMP and a
labelled (eg radiolabelled) cAMP or cAMP analog for a
high affinity binding site either on an immunoglobulin
or on a naturally occurring cAMP binding protein, in
particular cAMP dependent protein kinase isoenzyme type
I (cAPKI).

While such assays have been available for some
decades now, they are nonetheless less accurate than is
desirable and the antibody based assays are unduly
sensitive to the agents (eg EDTA) that are added to cAMP
containing samples to prevent degradation of cAMP before
the assay can be carried out.

There is thus a need for an improved assay for
cAMP.

We have now found that such an improved assay can

be achieved by using as a cAMP binding agent a
polypeptide comprising in functional form only one type
of cAMP binding site of a cAMP dependent protein kinase
(cAPK). cAPK, which exists as type I (cAPKI) and type
II (cAPKII), has two types of cAMP binding sites, the A
5 and B sites, on each of the regulatory (R) subunits
located respectively towards and at the C-terminus.
Thus for cAPKI, which is a dimer, there are four binding
sites Aα, Aβ, Bα and Bβ. In terms of amino acid
sequence the A sites on the α and β chains are identical
10 as are the B sites on the α and β chains.

cAMP dependent protein kinases are discussed for
example in Taylor et al. Ann. Rev. Biochem. 59: 971
(1990), Døskeland et al. Biochim. Biophys. Acta 1178:
249-258 (1993), Francis et al. Ann. Rev. Physiol. 56:
15 237-272 (1994) and Johnson et al. Chem. Rev. 101: 2243-
2270 (2001). The A and B sites of the polypeptide RIα
(the regulatory (R) subunit of the α chain of cAMP
dependent protein kinase type I) is described by Yu et
al. in Science 269: 807-813 (1995) who co-crystallized
20 cAMP and a truncated RIα with intact A and B sites. RI
has two forms, RIα and RIβ which are very similar in
terms of amino acid sequence in the cAMP binding
domains. The R subunits of cAMP dependent protein
kinase type II (i.e. RIIα and RIIβ) have A and B sites
25 that are similar to the corresponding sites in RIα.
(See Diller et al. Structure 9: 73-82 (2001)).

In mammalian R subunits, the A site comprises
roughly amino acid residues 143-260 (RIα) or 158-277
(RIIβ) and the B site comprises roughly residue 260-374
30 (RIα) or 277-426 (RIIβ).

Thus viewed from one aspect the invention provides
a method for assaying for cAMP in a sample, said method
comprising contacting a sample with an unknown cAMP
content with a polypeptidic cAMP binding agent and
35 optionally with a labelled cAMP, and detecting
conjugates of cAMP or labelled cAMP and said binding

agent, characterized in that said binding agent comprises functional cAPK cAMP B-binding sites only.

The functional site is a B site, more preferably a RI B-site, most preferably a RIα B-site.

By "functional site" is meant herein that the
5   binding site is capable of binding cAMP with a K_D of no more than 300% of that of the site in native human cAPK, preferably no more than 200%, more preferably no more than 150% and especially no more than 110%. (The lower the K_D, the higher the binding affinity).

10   Besides the functional cAMP binding site (or sites), the binding agent may contain non-functional cAMP binding sites, e.g. of a different type. By a non-functional site is meant a site corresponding to a native cAMP binding site in some or all of its amino
15   acid sequence but which has a K_D for cAMP binding which is more than 300% of that of the corresponding site in native human cAPK, preferably more than 500% and more preferably more than 10000%.

By a "cAPK cAMP binding site" is meant a
20   polypeptide sequence having the ability to bind cAMP and having a sequence homology with a native mammalian cAPK cAMP binding site sequence of at least 60%, preferably at least 80%, most preferably at least 95%. The cAPK cAMP binding site in the binding agent used in the assay
25   method of the invention thus need not be identical to a native mammalian cAPK cAMP binding site, e.g. due to insertions, deletions, substitutions or transpositions of one or more amino acids. The cAPK cAMP binding site in the binding agent will preferably contain at least
30   95% of the amino acid sequence of the corresponding cAPK cAMP binding site common to human and bovine cAPK, i.e. the highly conserved section of the sequence. The term "functional cAPK cAMP binding site" is also considered to extend to cAMP binding polypeptide sequences having a
35   functional topology equivalent to native cAPK cAMP binding sites, eg synthetic polypeptide sequences having

similar tertiary structure presenting the same or
equivalent functional groups within the cAMP binding
pocket.

As is routine in diagnostic assays, the detection
of the analyte:binding agent conjugate may be direct or
indirect. Thus for example where labelled cAMP is used
in the assay method a signal from the label in the
labelled cAMP:binding agent conjugate, for example a
radiation emission, may be detected directly and the
content of cAMP in the sample inferred from this.
Alternatively the signal from the label in the
uncomplexed labelled cAMP may be detected directly and
the content of cAMP in the sample again inferred from
this. As a further alternative, a secondary binding
agent which binds to cAMP:primary binding agent
conjugates or to uncomplexed primary binding agent and
which gives rise to a directly detectable signal or
event may be used and from the detected signal the
content of cAMP in the sample may again be inferred.
All such systems of analyte detection are encompassed by
the assay method of the invention.

Viewed from a further aspect the invention also
provides a kit for a cAMP assay, said kit comprising a
polypeptidic primary binding agent capable of binding
cAMP; optionally, a labelled cAMP; and optionally a
secondary binding agent; characterized in that said
primary binding agent comprises functional cAPK cAMP B-
binding sites only. The kit of the invention also
preferably includes instructions for the performance of
the assay method.

Viewed from a still further aspect the invention
provides a polypeptidic cAMP binding agent which
comprises functional cAPK cAMP B-binding sites only, and
compositions and items comprising said binding agent.

By compositions containing the binding agent are
included liquid, semi-solid (e.g. gel) and solid (e.g.
pulverulent) compositions, typically including further

substances such as liquid or solid carriers or gel
forming agents, preservatives, pH modifiers, etc. By
items comprising the binding agent are included for
example solid or semi-solid structures coated or
impregnated with the binding agent, e.g. beads, plates,
5   tubes, membranes, fibres, etc with the binding agent
immobilized thereon.

      The cAMP binding agent used according to the
invention is preferably a recombinant protein comprising
one or more, e.g. 1, 2 or 3, cAPK cAMP binding sites,
10   especially preferably bovine cAPK cAMP binding sites,
optionally a fusion protein comprising a section
couplable to a substrate surface (a "surface binding
region"). Where the binding agent contains two or more
cAPK cAMP binding sites, these should be of the same
15   type, thus for example both or all may be RIα B-sites.
The protein may, but need not necessarily, include a
disabled cAPK cAMP binding site. By disabled in this
context is meant that the amino acid sequence of the
cAMP binding site is modified to substantially reduce or
20   eliminate the cAMP binding ability of the site, e.g. by
substitution, insertion or deletion of one or more amino
acids so as to change the tertiary structure or the
functional topology of the site. In a preferred
embodiment, the binding agent thus has one functional RI
25   B-site and one disabled RI A-site. A-site disablement
is conveniently achieved by mutagenesis replacing a
highly conserved Gly residue (depending on the species
this may be residue 199, 200 or 201) in mammalian, e.g.
human or bovine, RIα by Glu. This can be done using PCR
30   and a standard mutagenesis kit (e.g. Strategene Quick
Change). The efficacy of A-site disablement can be
verified by equilibrium binding, e.g. using the method
of Hummel et al. BBA 63: 530-532 (1962) or by binding of
tritiated cAMP and precipitation with ammonium sulphate
35   as described further below. Especially preferably the
binding agent is a chimeric fusion protein containing

one or more cAPK B-sites, especially RIα B-sites, and a
polypeptide region which is one member of a specific
binding partner pair (e.g. a biotin binding site of
streptavidin) or which is a surface binding extension
(e.g. Gly Gly Cys or GST (glutathione S-transferase)).
Such chimeric fusion proteins comprising disabled cAPK
A-sites and one or more cAPK B-sites may readily be
prepared by conventional techniques, eg transfection of
bacterial hosts such as E. coli with plasmids
incorporating in an operable reading frame an
appropriately coded nucleic acid sequence. In an
especially preferred embodiment of the invention the
binding agent comprises a B-site modified relative to a
native mammalian cAPK B-site by replacement of Cys by
Ala or by another amino acid residue that cannot form a
disulphide bridge. This can be done by mutagenesis as
discussed above in relation to A-site disablement. Such
binding agents containing a Cys-free B-site are more
stable on storage than binding agents with Cys residues
in the B-site.

        The binding agent is preferably used in aqueous
solution under non-oxidizing conditions; typically the
solution will contain one or more chelating agents such
as EDTA and EGTA as stabilizers, and a reducing agent
such as DTE, DTT, 2-mercaptoethanol or glutathione.

        Where the assay method of the invention involves
competition for binding between the cAMP in the sample
and a labelled cAMP, it is preferred that the label be
tritium (^{3}H) and/or that the label be attached to a
substituent at the 8-position, i.e. as a substituent on
the adenosine ring structure. cAMP 8-labelled with ^{125}I
(generally attached via a linker group) is novel and
forms a further aspect of the present invention.

        ^{3}H-cAMP is available commercially from Amersham plc,
UK.

        The label in the labelled cAMP may be any species
which is detectable (e.g. by virtue of radiation

emission or absorption) or which can generate a
detectable species or event (e.g. by virtue of enzymatic
activity). Radiolabels, chromophores and fluorophores
are preferred as the label may be placed so as not to
interfere with or to minimally interfere with the

5   ability of the labelled cAMP to bind to the cAMP binding
agent. In one embodiment of the invention the labelled
cAMP is surface-bound cAMP with the conjugates formed by
binding of the cAMP binding agent to the surface-bound
cAMP being detectable for example by surface plasmon

10   resonance. Such surface binding of cAMP may be effected
for example by reacting a substrate having surface
carboxyls or activated carboxyls with an
8-aminoalkanoylamino-cAMP (e.g. 8-aminooctanoyl amino-
cAMP, available from BioLog, Bremen, DE). In this

15   embodiment the linker between the surface (e.g. the
polymer backbone of a polymeric substrate) and the
pendant cAMP groups preferably has a molecular weight of
less than 1000D, more preferably a molecular weight of
100 to 500D. (In this way the SPR signal difference on

20   binding of the binding agent to the surface is
optimised.) In another embodiment of the invention the
labelled cAMP is a polymer (e.g. a dendrimer) with
pendant cAMP groups (preferably 8-attached). Labelled
cAMP:binding agent conjugates can in this embodiment be

25   detected by light scattering (e.g. turbidimetry or
nephelometry). Where the labelled cAMP is radiolabelled
it is preferably ^{3}H-cAMP (detectable by scintillation) or
8-(^{125}I-X)-cAMP (where X is an organic group providing a
chain of up to 25 atoms to link the iodine to the 8-

30   position, e.g. a tyrosine-alkanoylamino group).
Alternatively ^{32}P-labelled cAMP (available from ICN
Pharmaceuticals) may be used. Preparation of labelled
cAMP can be effected by conventional chemical synthesis
techniques. Thus for example 8-amino-octanoyl-amino

35   cAMP (available from BioLog, Bremen, DE) may be reacted
with tyrosine to introduce a tyrosine residue at the 8-

position. This can be iodinated with ^{125}I using a
standard kit (e.g. operable to effect peroxidase
catalysed iodination using an inorganic ^{125}I compound).
The bulky substitution of the 8-position does not appear
to inhibit binding of the cAMP to a cAPK cAMP binding

5   site.

In one especially preferred embodiment of the
invention, conjugates of cAMP and of labelled cAMP with
the cAMP binding agent are precipitated and taken up by
a filter, e.g. a membrane filter. In this embodiment,

10   the conjugate precipitation is conveniently effected
using a sulphate solution, e.g. aqueous ammonium
sulphate. The cAMP binding sites of cAPK do allow for
cAMP exchange (i.e. complex dissociation) and so it is
desirable to "freeze" the quantity of labelled cAMP in

15   the precipitate. This can readily be done by the use of
a precipitation agent comprising a solution of cAMP and
sulphate. The solution is preferably almost sulphate
saturated, e.g. 55-95% saturated, more especially 75-92%
saturated and preferably contains cAMP in a quantity in

20   excess of the labelled cAMP in the sample to which it is
to be added, eg at a 0.1 mM concentration. In general
this "stop" solution will be added cold in a volume
ratio of about 20:1 to 1:2, more preferably about 15:1
to 5:1 relative to the sample. Typically the stop

25   solution is applied at temperatures below 10°C, e.g.
0°C, and if there is a delay between stop solution
application and separation of the precipitated conjugate
from the supernatant, the sample should be held cold
during this delay period, e.g. at 0°C. The stop

30   solution is preferably buffered, e.g. at pH7.

In this embodiment, following conjugate
precipitation, the precipitate and supernatant are
preferably separated by filtration, e.g. over a
micrometer range pore size filter (e.g. 0.45 μm pore

35   size). Suitable filter membranes are available
commercially from Millpore, e.g. HAWP or more preferably

HAMK filters.

After filtration, the filter membrane is preferably rinsed to remove uncomplexed labelled cAMP. This is preferably done immediately after filtration and is preferably repeated, e.g. one or two further rinses. Diluted stop solution may be used as the rinse solution; generally however this need not contain cAMP.

Where the sample has a high salt content, filtration should be effected as soon as possible after precipitation, e.g. within 30 minutes.

Where the sample under investigation has a low binding agent concentration, it is desirable to add casein to the stop solution to promote precipitation. Where the sample under investigation has a high binding agent concentration, it may be desirable to use glass fibre pre-filters.

Following precipitation and rinsing, the quantity of labelled cAMP retained by the filter membrane may be detected. In the case of chromophore or fluorophore labelling or labelling with radiolabels other than those requiring scintillation counting, the signal may be read from the membrane or following release of the conjugate or the labelled cAMP from the membrane. Where detection involves scintillation counting, e.g. as with tritium labelled cAMP, the conjugate or the labelled cAMP must be released from the membrane. This may conveniently be effected by contacting the membrane with an aqueous surfactant solution, e.g. a sodium dodecyl sulphate solution, typically a 1-5% w/v solution, especially a 2% w/v solution. The conjugate or the labelled cAMP released into solution may then be detected in the solution, e.g. in the case of ^{3}H-cAMP by addition of a scintillation fluid (e.g. "Emulsifier safe for aqueous samples" available from Packard).

As with most diagnostic assays, the assay system for use according to the invention will generally require calibration against standards (i.e. cAMP

solutions) with a range of known cAMP concentrations.
The assay kit of the invention will thus typically be
supplied with a set of standards for calibration and/or
a calibration chart and/or a computer program or dataset
for interpolation or extrapolation from signal values

5   for standards to determine the cAMP content of the
sample under investigation.

     The sample investigated using the method of the
invention will typically be of a biological or
biologically derived material, e.g. microorganisms,

10   cells, tissues, body fluids, body organs, etc. Of
particular interest are samples of or derived from
microorganisms, especially bacteria and yeasts. Also of
interest are samples of or derived from multicellular
organisms, e.g. mammals, reptiles, birds and fish,

15   especially humans. It should be noted however that cAMP
values will generally be lower in cell-free body samples
than in body cells, e.g. about nanomolar levels in serum
as opposed to micromolar levels in intracellular fluids.

     In many biological samples, once removed from the

20   host species the cAMP content reduces rapidly over time.
For this reason, the samples used in the assay method of
the invention will generally be pretreated to reduce or
eliminate cAMP degradation. Typically this may involve
addition of a buffer and/or a chelating agent (e.g.

25   EDTA). For assays of body tissue or organ samples,
since cAMP levels can drop very rapidly on sample
extraction or on the subject's death or exposure to
stress, it may be desirable to freeze the sample as
quickly as possible after extraction, e.g. by plunging

30   into liquid nitrogen. The sample may then be processed
further, e.g. ground, solvent extracted, etc, before
assay performance.

     The method of the invention is a method of assaying
for cAMP. This may involve generation of a

35   quantitative, semiquantitative or qualitative result,
e.g. the concentration of cAMP in the sample, the

concentration of cAMP relative to another analyte,
allocation of the result to a band in a multi-band
representation of cAMP concentration, indication of the
cAMP concentration as being above (or below) a
predetermined threshold value (e.g. one indicative of a
normality/abnormality boundary or a mild/severe
boundary), or even an indication of a predicted outcome,
e.g. the period for which a whole blood sample may
continue to be stored under refrigeration while still
being suitable for use in a transfusion. (For the
latter, the sample tested will generally be lysed
citrated whole blood or red blood cell concentrate).
All such determinations of cAMP content are considered
to fall within the assay method of the invention.

Besides assaying for cAMP, the assay method of the
invention may be used to assay for other cyclic
nucleotides (e.g. cGMP) and cyclic nucleotide analogs
that are capable of binding to the cAPK cAMP binding
site. If such assays are competitive assays, then the
competing labelled analyte may be a labelled cAMP or,
more preferably, a labelled version of the desired
analyte. Such assays are of particular importance in
the investigation of the biodistribution and
pharmacokinetics of candidate or actual cyclic
nucleotide (analog) drug compounds or precursors.
Unlike the conventional cAMP assays referred to above,
the assay method of the invention is sensitive and
accurate enough to detect such compounds. In such
assays, if desired, the sample may be treated to reduce
or eliminate any cAMP content which might interfere,
e.g. by treating the sample with an agent to which cAMP
binds but to which the candidate or drug does not or
which serves to transform cAMP into a species which does
not bind at the cAPK cAMP binding site, e.g. an enzyme
with phosphodiesterase activity which serves to
transform cAMP (but not the candidate or drug) into a
species which does not bind significantly at the cAPK

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cAMP binding site, or an antibody which binds cAMP but
not the candidate or drug.

Examples of cyclic nucleotides and analogs which
can be assayed for in this way include 8-
aminohexylamino-cAMP, 8-bromo-cAMP, 8-chloro-cAMP, 8-
5   chlorophenylthio-cAMP, N6-monobutyryl-cAMP and cGMP.

Thus viewed from a further aspect the invention
provides a method for assaying for a cyclic nucleotide
or cyclic nucleotide analog, said method comprising
contacting said sample with a polypeptidic binding agent
10   capable of binding said cyclic nucleotide or cyclic
nucleotide analog and optionally also with a labelled
competitor species capable of binding to said binding
agent, and detecting conjugates of said binding agent
with said cyclic nucleotide or cyclic nucleotide analog
15   or said competitor species, characterized in that said
binding agent comprises functional cAPK cAMP B-binding
sites only.

Viewed from a still further assay the invention
also provides a kit for an assay for a cyclic nucleotide
20   or cyclic nucleotide analog, said kit comprising a
polypeptidic primary binding agent capable of binding
said cyclic nucleotide or cyclic nucleotide analog;
optionally a labelled competitor species capable of
binding to said binding agent; and optionally a
25   secondary binding agent; characterized in that said
primary binding agent comprises functional cAPK cAMP B-
binding sites only.

This kit and assay method will preferably utilize
the features mentioned above in connection with the cAMP
30   assay and kit.

The assays of the invention, unlike the prior art
antibody-based assays, are insensitive to the presence
of divalent metal ions up to relatively high
concentrations (e.g. 10 mM). This is of importance
35   since the labile concentrations of such metal ions (e.g.
Ca^{2+} and Mg^{2+}) are strongly affected by the presence of

the chelating agents which are required to stabilize cAMP concentrations in samples before the assays are performed.

All publications referred to herein are hereby incorporated by reference.

5   The assay method of the invention will now be described further by reference to the following non-limiting Examples.

Example 1

10   ### cAMP Binding Agent Preparation

The NcoI blunt end fragment of the cDNA for a fusion polypeptide of GST and non-mutagenized human RIα containing a thrombin cleavage site between GST and RIα

15   was inserted in the NcoI-HindIII sites of plasmid pGEX-KG, the insert corresponding to nucleotides 103 to 1474 of the published sequence.

For mutagenesis a QuickChange Site-Directed Mutagenesis

20   Kit (available from Stratagene) was used. The RIα encoding double stranded plasmid and two synthetic oligonucleotide primers with the desired mutations were annealed and extended by means of the *Pfu* DNA polymerase. The synthetic oligonucleotide primers were

25   5'-GGAGGGAGCTTTGAAGAACTTGCTTTG and 3'-CCTCCCTCGAAACTTCTTGAACGAAAC. After temperature cycling, the parental DNA template was digested using *Dpn* 1. The mutated DNA was transformed into Epicurian Coli ® XL1-Blue supercompetent cells. The mutations were confirmed

30   by sequencing (ABI Prism 3700).

The plasmid was transformed into E. coli BL21, and preincubated for 2 hours before expression was induced with 0.4 mM IPTG. The bacterial incubation was

35   performed at 25°C.

The expressed GST hRIα fusion protein was purified by
binding to glutathione-agarose (Pharmacia), according to
the manufacturer's protocol. The fusion protein was
further purified on a Superdex 200 gel filtration column
(Pharmacia FPLC system). The GST-tag was cleaved off by
5   thrombin followed by an additional FPLC size exclusion
chromatography step.

SDS-PAGE mobility of GST-hRIa fusion protein
corresponded to the expected value of 81 kDa using
10   bovine serum albumin, hen ovalbumin and Bio-Rad high
molecular weight standards for calibration.

The thrombin cleaved protein had SDS-PAGE mobility of 51
kDa.
15

Example 2
Assay Performance

Sample preparation
20   Male Wistar rats (120-400 g) were anaesthetized, the
liver exposed, and a biopsy snap-frozen between the
metal clamps of a Wollenberger tong precooled in liquid
nitrogen. The frozen tissue was pulverized. (The
powder could be stored in liquid nitrogen for months
25   without cAMP degradation). To extract cAMP the powder
was precipitated in ice-cold 5% (w/v) aqueous
trichloroacetic acid in 0.1 M HCl (1 ml per 50 mg tissue
powder), and centrifuged (20,000xg av.) for 10 minutes.
The trichloroacetic acid was removed from the
30   supernatant by repeated (4x) extraction with at least 4
volumes of water-saturated diethyl ether. The sample
was neutralized by the addition of 20 µl of 10 M NaOH.

Assay performance
35   100 µL of sample is mixed with 50 µL of 3 nM ^{3}H-cAMP
(Amersham plc, UK) and then with 50 µL of the thrombin

cleaved binding agent of Example 1 (1.2 nM in cAMP
binding sites) in 50 mM HEPES (adjusted to pH 7.4 with
dipotassium phosphate and containing 20 mM EDTA, 3 mM
EGTA, 0.5 mg/mL serum albumin, 0.2 mg/mL soybean trypsin
inhibitor and 0.5 mM DTE). The mixture is incubated for
2 to 18 hours and then mixed vigorously with 1 mL ice
cold 80% saturated ammonium sulphate. · `

2 x 2 mL of ice cold 65% saturated ammonium sulphate is
passed through a 25 mm diameter membrane filter (0.45 μm
pore size, e.g. HAMK available from Millipore)
whereafter the precipitated sample is passed through the
same filter under suction. Immediately after the sample
has run through, 2 mL of ice cold 65% saturated ammonium
sulphate are applied followed by a further two rinses
with 2 mL of ice cold 65% saturated ammonium sulphate.

The filter is transferred to a scintillation vial
containing 1 mL of 2% w/v aqueous sodium dodecyl
sulphate solution. The precipitate is dissolved by
vortexing for 10 minutes. Thereafter 7 mL of
scintillation fluid ("Emulsifier Safe for aqueous
samples" from Packard) are added and the vial is counted
in a beta-counter for at least 4 minutes.

A calibration curve, prepared using the same assay
procedure and standard solutions of cAMP in 50 mM HEPES
(adjusted to pH 7.4 with dipotassium phosphate and
containing 20 mM EDTA, 3 mM EGTA, 0.5 mg/mL serum
albumin, 0.2 mg/mL soybean trypsin inhibitor and 0.5 mM
DTE) is used to determine the cAMP content of the
sample.

Example 3

Assay Performance

Where the sample contains a low quantity of cAMP or a
high concentration (e.g. 10 mM) of a weakly binding
substance (e.g. ATP or AMP) which can dissociate from
the binding agent post-incubation, the assay of Example
2 may be performed omitting the ^{3}H-cAMP from the initial
5   incubation and subsequently performing a one hour post
incubation in the presence of 10 nM ^{3}H-cAMP. The
procedure is otherwise unchanged.